The effects of epithelial rests of Malassez cells on periodontal ligament fibroblasts: A co-culture investigation for epithelial-mesenchymal interactions

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Abstract

Background: The major function of epithelial rests of Malassez (ERM) cells is to maintain the homeostasis of the periodontal ligament (PDL). The purpose of this study was to characterize the effects of ERM cells on PDL fibroblasts in vitro using a co-culture system. Methods: PDL fibroblasts and ERM cells derived from porcine tissues were used. PDL fibroblasts were seeded in 6-well dishes. ERM cells plated in a chamber with a 0.4 μm pore membrane, were placed into the wells for 5 days. Osteocalcin (OCN), bone sialoprotein (BSP), osteoprotegerin (OPG) and receptor activator of NF-κB ligand (RANKL) mRNA levels in the PDL fibroblasts were then analyzed using real time RT-PCR. Alkaline phosphatase (ALP) activity was also measured. PDL fibroblasts cultured without ERM cells were used as a control. Results: OCN, BSP and OPG mRNA levels in PDL fibroblasts co-cultured with ERM cells were lower than the levels in the control group. Meanwhile, the RANKL mRNA level in PDL fibroblasts co-cultured with ERM cells was significantly higher than that of the controls (P<0.01). ALP activity of PDL fibroblasts co-cultured with ERM cells was significantly lower than in the controls (P<0.01). Conclusion: This study shows that ERM cells affect the functions of PDL fibroblasts, which decrease hard tissue formation and increase bone resorption. Therefore, ERM cells prevent dento-alveolar ankylosis of the PDL.

Key words: Epithelial rests of Malassez cells; periodontal ligament; fibroblasts; co-culture techniques; RT-PCR

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Introduction

The periodontal ligament (PDL) is a unique connective tissue which surrounds the tooth root and is connected to the alveolar bone (1). The homeostasis of the PDL is thought to be maintained by epithelial rests of Malassez (ERM) cells and to control the relationship between the cementum and the alveolar bone (2, 3). ERM cells, which are clusters of epithelial cells derived from Hertwig’s epithelial root sheath (4), form a network of epithelial strands throughout the PDL (5). Furthermore, ERM cells have a number of distinct functions, such as preventing resorption of the tooth root (6), participating in elongation of the periodontal pocket (7), participating in periapical cyst formation (8), and inducing cementum formation (9). The width of the PDL is maintained by formation and absorption of the alveolar bone. The alveolar bone is an active remodeling compartment, which can adapt its shape to accommodate the PDL space in tooth development (10) and in physical tooth movement (11). ERM cells are located in the PDL near the root cementum. Therefore, it is thought that ERM cells might play an important role in maintaining the PDL space, and a previous study showed the possible roles of ERM cells in maintaining the PDL (12). Fujiyama et al. reported that decreased ERM cell function led to dento-alveolar ankylosis (13). Therefore, evaluation of the relationship between ERM cells and PDL fibroblasts is important for clarifying the mechanism(s) underlying maintenance of the width of the PDL.

The purpose of this study is to investigate and confirm the effects of ERM cells on PDL fibroblasts in vitro using a co-culture technique.
Materials and methods

PDL fibroblasts and ERM cells

Periodontal tissues were obtained from the premolars of swine, according to the method of Inoue et al. (14). PDL fibroblasts were then incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for primary culture, using alpha-minimal essential medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma, St Louis, MO, USA) supplemented with 2 ml gentamycin (10 mg/ml Sigma). The medium was changed every 48 hrs, and cells in the third subculture were used for the experiments.

ERM cells derived from porcine PDL were provided by Prof. Yoshihiro Abiko, Department of Dental Science, Institute of Personalized Medical Science at the Health Science University of Hokkaido. The method used to obtain ERM cells has been described by Liu et al. (15). ERM cells were cultured in the same medium used for PDL fibroblasts in 75 cm² tissue culture flasks (Corning, Tokyo, Japan) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. When cells became almost confluent, they were detached using 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA, pH 7.2), then were resuspended in the culture medium described above and used for experiments.

Co-culture system and mRNA expression

PDL fibroblasts were seeded at a density of 5.2 × 10³ cells per cm² in 6-well-dishes (Corning, Tokyo, Japan) and ERM cells were also seeded at a density of 1.2 × 10³ cells per cm² on cell culture inserts with 0.4 μm pores (BD Falcon, Franklin Lakes, NJ, USA). Figure 1 shows a scheme of the co-culture technique used. Co-cultured PDL fibroblasts and ERM cells were maintained in the above-mentioned medium for 5 days.

Total RNAs were extracted using the acid guanidium thiocyanate/phenol-chloroform method as follows: After 5 days of co-culture, ERM cells in the culture dishes and the remaining total RNA pellets were washed with 70% cold ethanol and dissolved in 50 μl RNAase-free (DEPC-treated) water. Total RNAs were reverse transcribed and amplified in 20 μl volumes using a Quanti-Tect Reverse Transcription Kit® (Qiagen Inc., Hilden, Germany) containing RNA PCR Buffer, 2 U/μl RNAase inhibitor, 0.25 U/μM reverse transcriptase, 0.125 μM oligo dt-adaptor primer, 5 mM MgCl₂ and RNAase-free water. RT-PCR products were analyzed by quantitative real-time RT-PCR in TaqMan Gene Expression Assays® (Applied Biosystems, Foster, CA, USA) for the following target genes: osteocalcin (OCN), bone sialoprotein (BSP), osteoprotegerin (OPG) and receptor activator of NF-κ B ligand (RANKL). The TaqMan Endogenous Control (Applied Biosystems) for the target gene β-actin was used as a control. Primer sequences used are shown in Table 1. All PCR reactions were performed using a real time PCR 7500 Fast System®. Gene expression quantitation using TaqMan Gene Expression Assays® was performed as the second step in a two-step RT-PCR. Assays were done in 20 μl singleplex reactions containing TaqMan Fast Universal PCR Master Mix®, TaqMan Gene Expression Assays®, distilled water and cDNA, according to instructions provided by the manufacturer (Applied Biosystems). Reaction conditions consisted of a primary denaturation at 95°C for 20 sec, then cycling for 40 cycles of 95°C for 3 sec and 62°C for 30 sec. PCR data are reported compared to the corresponding control. Quantitative RT-PCR analyses were reproduced 4 times.

Alkaline phosphatase activity assay

ALP activity was determined at 5 days after the culture. After removal of the culture medium from the dishes, the cells were rinsed with PBS. Distilled water was added and cells were harvested with a scraper and then transferred to 15

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**Table 1.** Primer sets used for quantitative RT-PCR analyses

| Gene | Forward Primer | Reverse Primer | Probe
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>VEGF</td>
<td>F:CATCTTCAAGCGGTCTGTTGT</td>
<td>R:CCAGACCTTCTGTTGCA</td>
<td>Pro:CCGCGACCCATCAG</td>
</tr>
<tr>
<td>BSP</td>
<td>F:CCTCACCCAGACATTCTCT</td>
<td>R:GGCCAGCTGTCGTTACATCT</td>
<td>Pro:CCACCTGTCCCTGTC</td>
</tr>
<tr>
<td>OCN</td>
<td>F:ACCAACAGACGCCACTCAG</td>
<td>R:GGCCAGACAAGGCAGAGT</td>
<td>Pro:CCCTACACTGCTTC</td>
</tr>
<tr>
<td>OPG</td>
<td>F:GCTGTCCTCTGAGATTGGA</td>
<td>R:CATCTGACATCTTCTGGCAACTGT</td>
<td>Pro:CCGGACGAAGCCTC</td>
</tr>
<tr>
<td>RANKL</td>
<td>F:CCATCGGTTCCCACAAAGT</td>
<td>R:GCCCAAACCTCGTCATGATA</td>
<td>Pro:CCAGCGAGACAGACTC</td>
</tr>
</tbody>
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![Fig. 1. Scheme of the co-culture system used in this study.](image-url) Periodontal ligament (PDL) fibroblasts were seeded in the lower compartment, and epithelial rests of Malassez (ERM) cells were seeded in the upper compartment, which has a microporous membrane (pore size: 0.4 μm) on the bottom.
mL plastic tubes. The cells were sonicated for 10 min and centrifuged at 2,000 rpm for 10 min, and the supernatants were measured for absorbance at 405 nm using an ALP analysis kit (Monotest ALP opt®, Boehringer Mannheim, Darmstadt, Germany). All procedures were carried out on ice.

To quantify ALP activity, protein concentrations in those supernatants were determined using a protein assay kit (Coomassie Plus Protein Assay Reagent Kit® Takara Bio Inc. Otsu, Japan). A 150 μl aliquot of each supernatant as described above was added to 150 μl of the Coomassie reagent in a 96-well culture plate. After mixing the plate for 30 sec, absorbance was measured at 595 nm using a microplate reader (iMark®, Bio-Rad Laboratories, Inc., Hercules, CA, USA). These operations were also carried out on ice.

Enzymatic activities were finally determined by absorbance values of reaction products per min according to the following formula:

\[
\text{ALP specific activity (IU/μg)} = \frac{\text{ALP concentration (IU/mL)}}{\text{protein concentration (μg/mL)}}
\]

**Statistical analysis**

Data were analyzed via one-way ANOVA and were compared by Scheffe’s test.

**Results**

The OCN mRNA expression by PDL fibroblasts co-cultured with ERM cells was approximately 1.8 times lower than by PDL fibroblasts cultured without ERM cells \((P<0.01)\) (Fig. 2). The BSP mRNA expression by PDL fibroblasts co-cultured with ERM cells was approximately 1.25 times lower than by PDL fibroblasts cultured without ERM cells \((P<0.05)\) (Fig. 3). Furthermore, OPG mRNA expression by PDL fibroblasts co-cultured with ERM cells was approximately 3.4 times lower than by PDL fibroblasts cultured without ERM cells \((P<0.01)\) (Fig. 4). RANKL mRNA expression by PDL fibroblasts co-cultured with ERM cells was significantly higher than by PDL fibroblasts cultured without ERM cells \((P<0.01)\) (Fig. 5).

ALP activity in PDL fibroblasts co-cultured with ERM cells was 4.871 ± 0.198 × 10⁻⁷ μM/μg/sec, but was 5.405 ± 0.415 x 10⁻⁷ μM/μg/sec in PDL fibroblasts cultured without...
ERM cells, which was a statistically significant difference ($P<0.01$) (Fig. 6).

**Discussion**

To demonstrate functional interactions between ERM cells and PDL fibroblasts, co-cultures of ERM cells and PDL fibroblasts without other types of cells is a useful approach. Inoue et al. reported interactions between ERM cells and PDL fibroblasts using co-cultures in vitro, but they cultured ERM cells and PDL fibroblasts that were mixed in the bottom of each culture dish (14). In contrast, our study used a co-culture system in which a membrane with 0.4 μm pores allowed the exchange of medium, including factors secreted by ERM cells, but cell-to-cell interactions between ERM cells and PDL fibroblasts were inhibited (16). Using this co-culture system to characterize the effects of ERM cells on PDL fibroblasts is very convenient, and this study is the first such trial reported in the literature.

It is well known that PDL fibroblasts have a function in hard tissue formation. On the root surface, cellular and acellular cementum is present, and ERM cells are also located in the PDL tissue near the root cementum (17). Therefore, it may be speculated that the cementum and/or the epithelium might play an important role in maintaining the periodontal space (13).

OCN and BSP mRNAs are markers for bone and other kinds of hard tissue formation. In addition, ALP activity is a marker for osteogenic cell differentiation. In this study, those markers in PDL fibroblasts co-cultured with ERM cells were significantly lower than in PDL fibroblasts cultured without ERM cells. This means that ERM cells inhibit the differentiation of PDL fibroblasts to osteogenic cells. On the other hand, OPG and RANKL are secreted by osteoblasts and fibroblasts to recruit macrophages and to stimulate the differentiation to osteoclasts (18). This study shows that OPG and RANKL mRNA levels in PDL fibroblasts co-cultured with ERM cells were significantly lower than those of PDL fibroblasts cultured without ERM cells. Thus, ERM cells promote PDL fibroblasts to secrete OPG and RANKL, which promotes the appearance of osteoclasts.

In conclusion, this study demonstrates that ERM cells reduce the functions of hard tissue formation of PDL fibroblasts and increase bone resorption. Thus, ERM cells prevent the PDL from being susceptible to dento-alveolar ankylosis.

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**References**

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